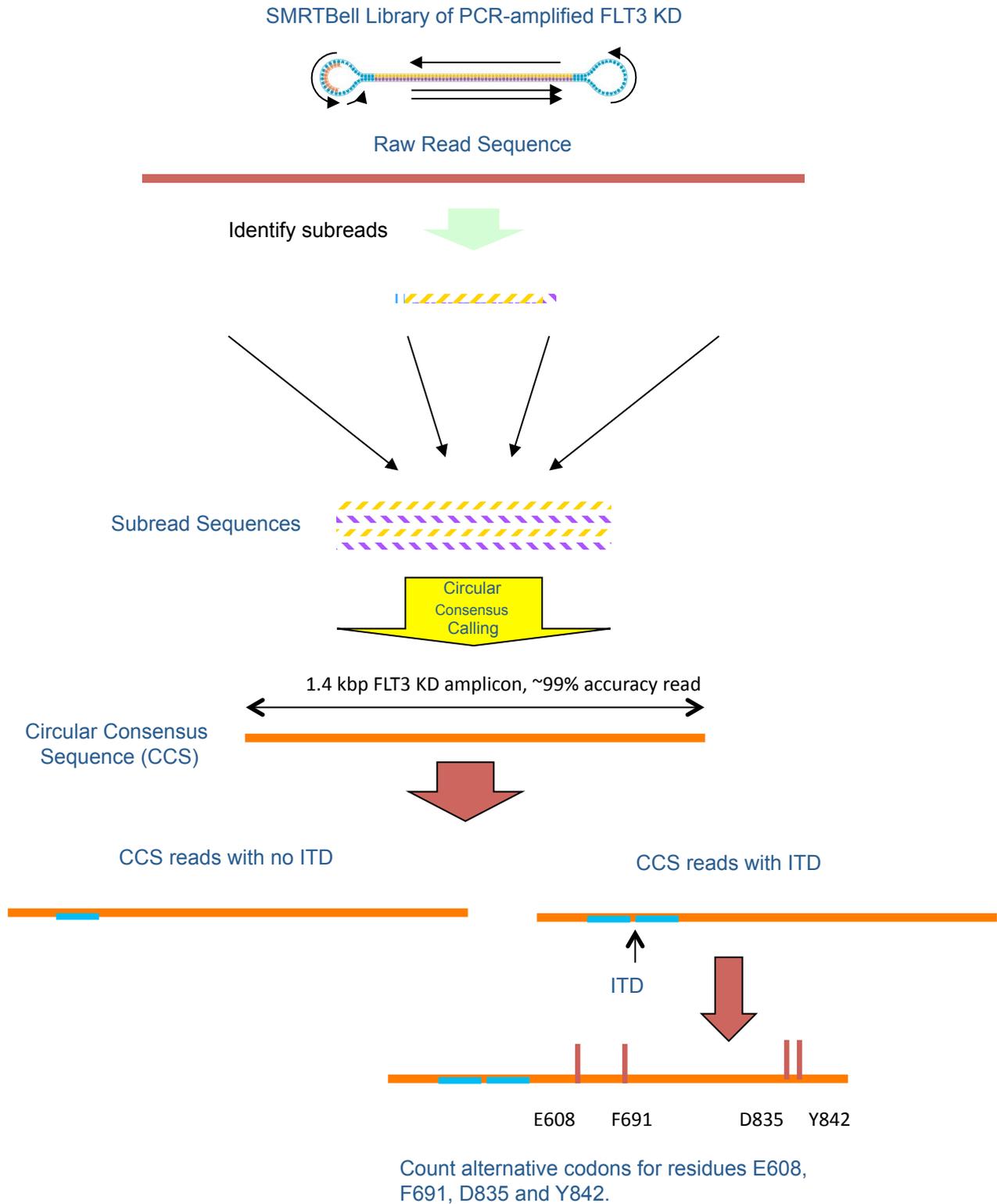
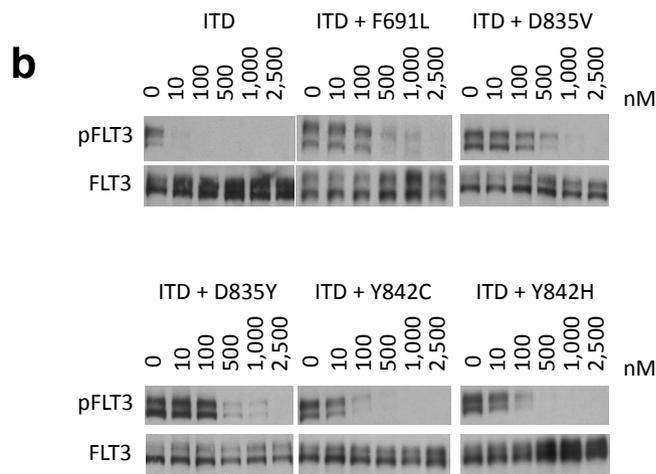
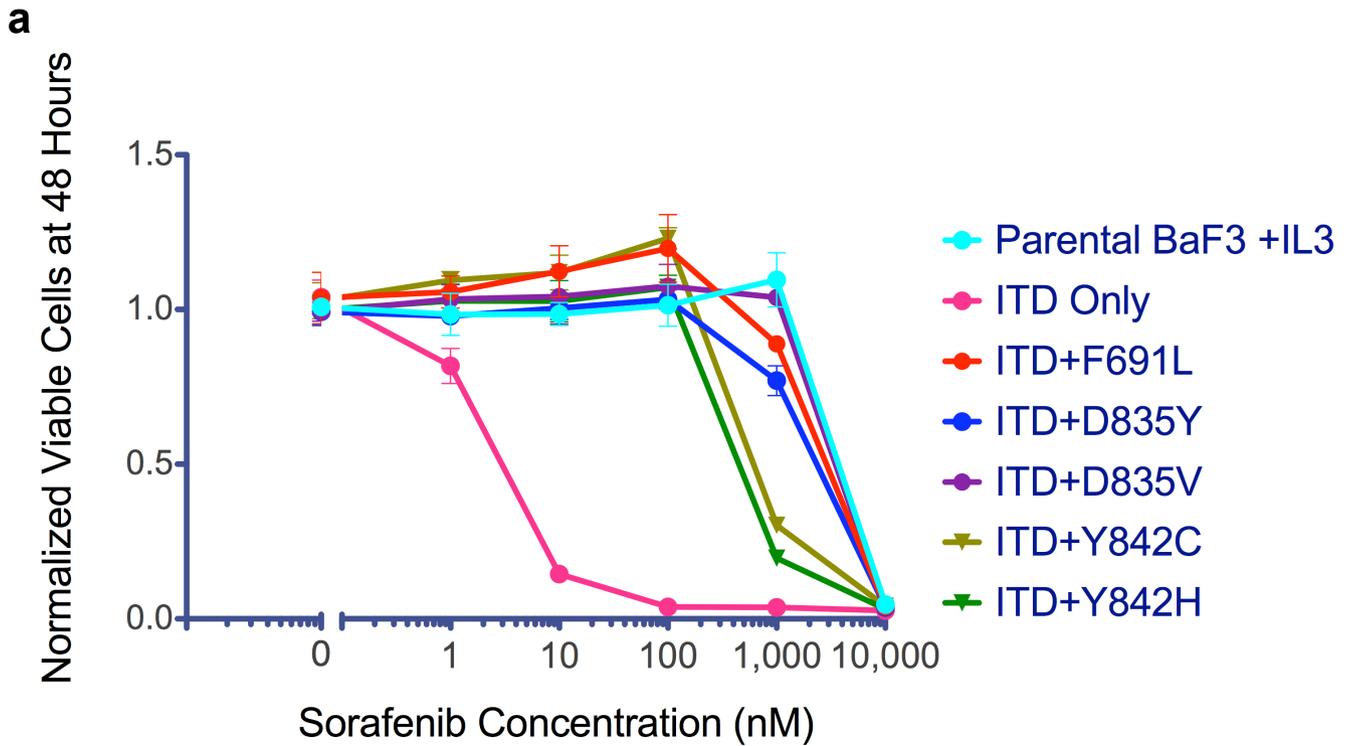


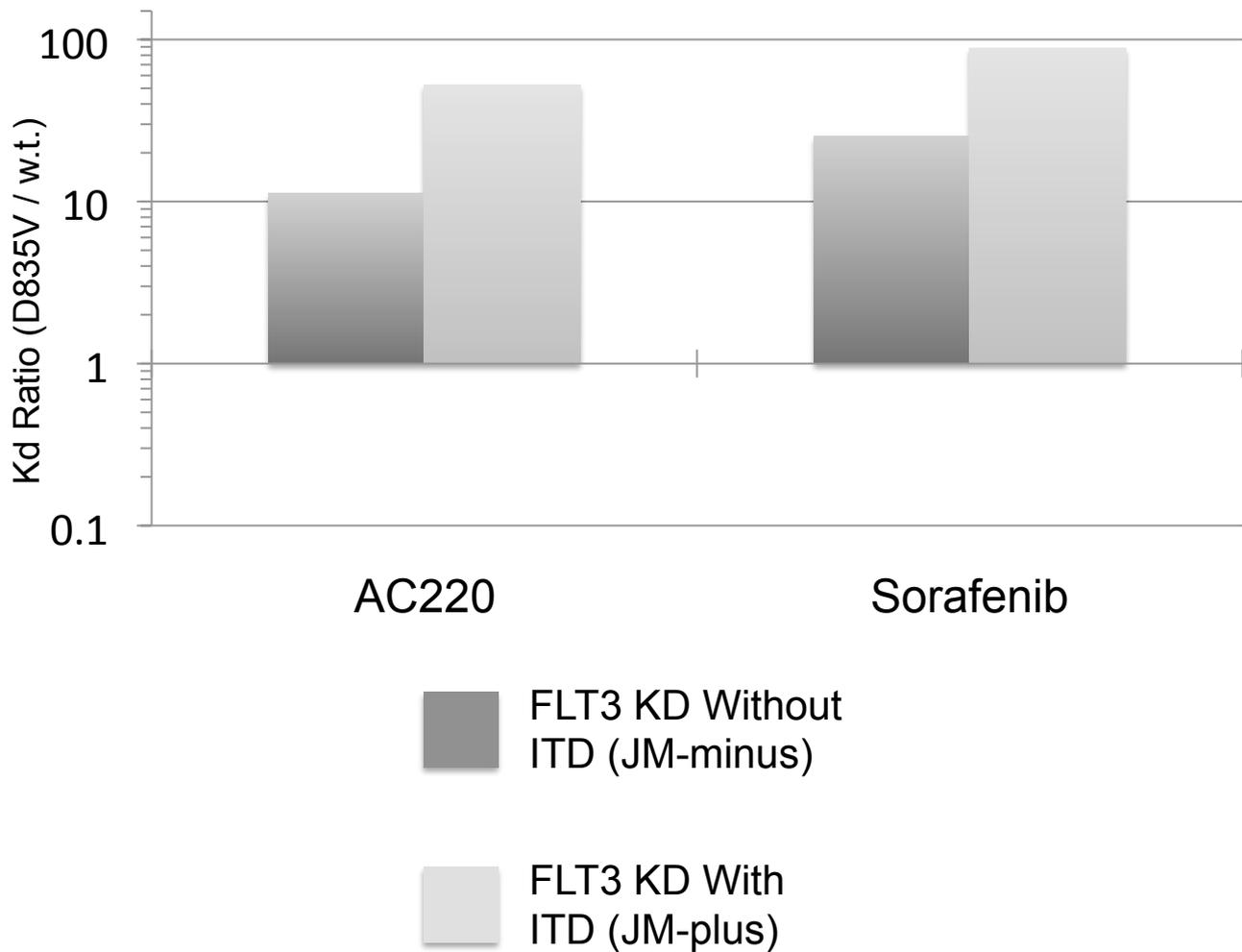
Supplementary Figure 1. D835F Mutation Confers Resistance to ACC220 *in vitro*. **a**, Normalized cell viability of Ba/F3 populations stably expressing FLT3-ITD or FLT3-ITD/D835F after 48 hours in various concentrations of AC220 (error bars represent s.d. of triplicates from the same experiment). **b**, Western blot analysis using anti-phospho-FLT3 and anti-FLT3 antibodies performed on lysates prepared from IL-3-independent Ba/F3 populations infected with retroviruses expressing FLT3-ITD and FLT3-ITD/D835F. Cells were exposed to the concentrations of AC220 indicated for 90 minutes.



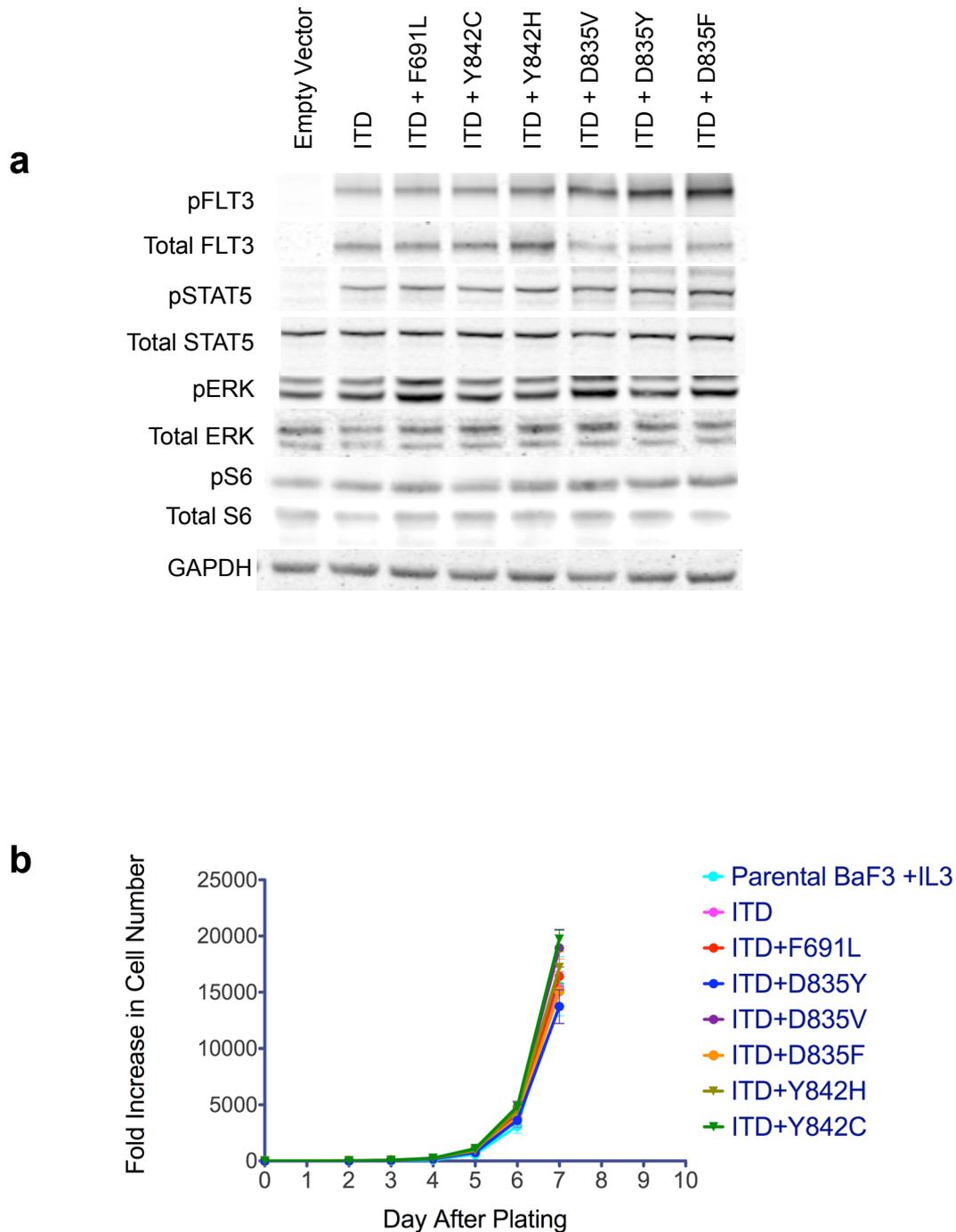
Supplementary Figure 2. Generating Accurate Circular Consensus Reads from SMRTbell Molecules. Overview of workflow for data analysis of SMRT sequencing of the FLT3 JM and KD regions using circular consensus sequence calling.



Supplementary Figure 4. AC220-resistant FLT3-ITD Mutant Isoforms Confer Cross-Resistance to Sorafenib *in vitro*. **a**, Normalized cell viability of Ba/F3 populations stably expressing AC220-resistant FLT3-ITD mutant isoforms after 48 hours in various concentrations of sorafenib (error bars represent s.d. of triplicates from the same experiment). **b**, Western blot analysis using anti-phospho-FLT3 or anti-FLT3 antibody performed on lysates prepared from IL-3-independent Ba/F3 populations infected with retroviruses expressing the FLT3-ITD mutant isoforms indicated. Cells were exposed to the concentrations of sorafenib indicated for 90 minutes.



Supplementary Figure 5. Binding Assay Demonstrates AC220 and Sorafenib Bind Unfavorably to the D835V Mutation Both in the Presence and Absence of the ITD. Graphical representation of ratio comparison of binding constant (K_d) for drug binding to D835V mutant/native FLT3. w.t. = wild type.



Supplementary Figure 6. FLT3 Mutant Isoforms Display Differing Levels of FLT3 Auto-phosphorylation But Minimal Difference in Phosphorylation of Downstream Targets or Proliferation. **a**, Western blot analysis using anti-phospho-FLT3 and anti-FLT3 antibodies performed on lysates prepared from 293T cells transfected with FLT3 mutant isoforms. **b**, Proliferation of Ba/F3 stably expressing FLT3 mutant isoforms over a 7 day period. Proliferation expressed as fold increase over cell count at Day 0 (error bars represent s.d. of triplicates from the same experiment).

Supplementary Table 1. Mutations Found in Relapse Samples are Not Identified by Subcloning and Sequencing of FLT3 in Pre-Treatment Samples.

Subject Number	New Mutation at Relapse	Pre-Treatment ITD+ Clones with Mutation
1009-003	D835F	0/13
1011-006	D835Y	0/12
1011-007	F691L	0/11
	D835V	0/11
1005-004	F691L	0/22
1005-006	D835Y	0/15
1005-007	D835V	0/11
1005-009	D835Y	0/11
1005-010	F691L	0/24

Supplementary Table 2. Number of Circular Consensus Sequence (CCS) Reads Containing the ITD and Region of Interest

Sample	Total Aligned CCS reads	Average CCS Read Aligned Length	ITD+/-	Number of CCS Reads Aligned to the ITD and Flanking Region (bp 1750 to 2150)	Average Length of CCS Reads Aligned to ITD and Flanking Region	Number of CCS reads Spanning through the ITD and Y842 (bp 1750 to 2612)	Average Length of CCS reads Spanning ITD and Y842	Average Alignment Identity (%)
1009-003 Pre-Treatment	1223	1344.64	ITD-	604	1366	593	1376	98.2
			ITD+	561	1386	553	1395	96.8
1009-003 Relapse	963	1319.25	ITD-	399	1369	395	1376	98.2
			ITD+	496	1376	486	1389	96.6
1011-006 Pre-Treatment	724	1361.83	ITD-	480	1363	473	1373	99.1
			ITD+	217	1455	217	1455	93.5
1011-006 Relapse	1600	1325.39	ITD-	975	1358	957	1371	99.1
			ITD+	506	1448	503	1452	93.3
1011-007 Pre-Treatment	2400	943.69	ITD-	478	1342	467	1355	99.2
			ITD+	1057	1361	1033	1376	98.0
1011-007 Relapse	697	1339.89	ITD-	75	1372	74	1382	99.1
			ITD+	582	1383	572	1394	97.7
1005-004 Pre-Treatment	978	1251.51	ITD-	1	1393	1	1393	99.5
			ITD+	819	1416	805	1426	94.8
1005-004 Relapse	1900	1094.73	ITD-	4	1381	4	1381	99.1
			ITD+	1363	1400	1343	1409	94.3
1005-006 Pre-Treatment	804	969.71	ITD-	321	1366	318	1371	99.0
			ITD+	200	1429	199	1432	94.9
1005-006 Relapse	1825	976.82	ITD-	732	1370	715	1387	97.0
			ITD+	383	1412	378	1420	94.1
1005-007 Pre-Treatment	1306	1170.97	ITD-	996	1344	980	1355	98.9
			ITD+	62	1429	62	1429	93.9
1005-007 Relapse	1341	1080	ITD-	357	1330	348	1345	99.1
			ITD+	600	1403	589	1415	93.7
1005-009 Pre-Treatment	4856	174.66	ITD-	493	1357	484	1369	98.2
			ITD+	22	1425	22	1425	93.8
1005-009 Relapse	786	1157.96	ITD-	17	1322	16	1357	98.6
			ITD+	582	1434	576	1439	93.7
1005-010 Pre-Treatment	912	1221.58	ITD-	2	1310	2	1310	98.6
			ITD+	774	1368	757	1383	97.3
1005-010 Relapse	769	908.98	ITD-	1	1459	1	1459	94.4
			ITD+	476	1354	463	1372	97.3
Normal Control #1	1141	1102.94	ITD- ITD+	899	1338	886	1347	99.2
Normal Control #2	655	579.23	ITD- ITD+	150	1327	146	1347	99.3
Normal Control #3	1327	1000.02	ITD- ITD+	950	1351	939	1357	99.3

Supplementary Table 3. Mutations at Residues D835, F691, E608 and Y842 in FLT3 are Not Identified in Normal Controls

Sample	Residue Position	Observed Codon	Amino Acid	Number of Observations of Alternative Codon	Number of Observations of Reference Codon	Total Number of Sequences Sampled	Codon Frequency	p-value (Compared to Normal #1)
Normal Control #2	608	aga	R	0	92	92	0.00%	4.16E-01
	608	gag	E	0	92	92	0.00%	4.16E-01
	608	gga	G	0	92	92	0.00%	5.11E-01
	608	gaa	E	-	92	92	100.00%	1.00E+00
	691	ttc	F	1	83	85	1.20%	6.82E-02
	691	ttg	L	1	83	85	1.20%	1.22E-01
	691	ttt	F	-	83	85	97.60%	1.00E+00
	835	ggt	G	0	118	118	0.00%	3.54E-01
	835	gtt	V	0	118	118	0.00%	3.54E-01
	835	gac	D	0	118	118	0.00%	5.17E-01
	835	gat	D	-	118	118	100.00%	1.00E+00
	842	tgt	C	1	105	106	0.90%	5.36E-02
	842	tac	Y	0	105	106	0.00%	5.94E-01
	842	tat	Y	-	105	106	99.10%	1.00E+00
	Normal Control #3	608	aaa	K	1	538	540	0.20%
608		aga	R	0	538	540	0.00%	6.04E-01
608		gag	E	0	538	540	0.00%	6.04E-01
608		gga	G	1	538	540	0.20%	6.71E-01
608		gaa	E	-	538	540	99.60%	1.00E+00
691		att	I	2	515	518	0.40%	6.28E-01
691		ttc	F	1	515	518	0.20%	1.00E+00
691		ttg	L	0	515	518	0.00%	6.01E-01
691		ttt	F	-	515	518	99.40%	1.00E+00
835		aat	N	1	805	820	0.10%	1.00E+00
835		cat	H	1	805	820	0.10%	1.00E+00
835		gtg	V	1	805	820	0.10%	1.00E+00
835		ggt	G	3	805	820	0.40%	6.88E-01
835		gtt	V	8	805	820	1.00%	6.59E-02
835		gac	D	1	805	820	0.10%	4.41E-01
835		gat	D	-	805	820	98.20%	1.00E+00
842		aat	N	1	721	726	0.10%	1.00E+00
842		cat	H	1	721	726	0.10%	1.00E+00
842		tac	Y	3	721	726	0.40%	7.44E-01
842		tat	Y	-	721	726	99.30%	1.00E+00

Bold = native codon

Supplementary Table 4. Calculated IC₅₀ Values for Proliferation of Ba/F3 cells Expressing FLT3 Mutant Isoforms in the Presence of AC220 and Sorafenib.

Mutation	IC ₅₀ (nM)	
	AC220	Sorafenib
ITD	0.23	2.3
ITD + F691L	128	2316
ITD + D835V	85	3227
ITD + D835Y	23	2081
ITD + Y842C	19	598
ITD + Y842H	21	449

Supplementary Table 5. Calculated K_d for Binding of AC220 and Sorafenib to FLT3 D835V Mutation in the Presence and Absence of ITD.

Assay	K_d (nM)	
	AC220	Sorafenib
FLT3-JMminus	1.3	13.0
FLT3(D835V)-JMminus	14.6	328
FLT3(ITD)-JMplus	8.8	79
FLT3(ITD,D835V)-JMplus	463	6959

JM = juxtamembrane domain